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PRINCIPAL INVESTIGATOR: Dave S.B. Hoon, Ph.D.

CONTRACTING ORGANIZATION: John Wayne Cancer Institute
Santa Monica, CA 90404

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ABSTRACT

As proposed, we have successfully established assays to detect free tumor-related DNA marker(s) in serum of prostate cancer (PCa) patients and studied the potential use of the biomarker detection as surrogate genetic markers in monitoring PCa patients. Despite slight delay in the beginning, the program has move expediently over the years from assay development and optimization to subject sample accrual and assessment. DNA markers involving allelic instability (AI; 6 markers) and methylation of genes (3 markers) with high specificity and sensitivity were screened and established. Of the 83 AJCC stage I-IV PCa patients studied, the proportion of patients demonstrating AI for $\geq$1 marker was 47% (38/81 patients). Methylation biomarkers were detected in 24/83 (28%) patients. By combining two DNA assays, the number of PCa patients positive for $\geq$1 methylated or AI marker increased to 52/83 (63%). The combined assays detected PCa in 15 of 24 (63%) patients with normal PSA levels. The combination of the DNA biomarker assays detected the presence of PCa regardless of AJCC stage of PSA level. The results obtained through this award demonstrate that tumor-related DNA marker serum assay may be used, independent of AJCC stage or PSA level, in indentifying and monitoring patients with PCa.

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INTRODUCTION

In the management of patients with prostate cancer (PCa) using currently available diagnostic methods, there are major deficiencies in accurately identifying the stage of disease, monitoring for early stages of disease recurrence, and detecting metastasis. Tumor-related nucleic acids can contain a wealth of information if deciphered and translated into clinicopathological relevance. In the proposal, the detection of tumor-related serum DNA is studied as potential surrogate markers for PCa disease presence and progression. Tasks completed during the program included accrual of subjects and samples, determination of assay feasibility, optimization of DNA isolation and modification, assessment of microsatellite and methylation markers, analysis of data, correlation of marker data and clinical data, and final reporting of results. In a manuscript currently submitted and in review, we are reporting findings from this proposal. Specificity and sensitivity of the detection assay was established; LOH and methylation status of the serum DNA was correlated to clinical data. In total of 83 PCa patients and 40 normal subjects, we found the serum assay with both AI and methylation markers to not only complement the current PSA serum assay but surpasses in detection of cancer in patients.

BODY

Although initially delayed by several human subject IRB issues, we are able to bring the proposed study to a satisfactory conclusion and complete this report. Sufficient numbers of participants from both cancer and cancer free populations were accrued through the joint efforts of our institute, Fred Hutchinson Cancer Center, and The Angeles Clinic, as described in the Statement of Work Task 1. The patients have been accrued from these three centers as outlined in the proposal.

Blood was processed as indicated in Task 1. The blood was purified and cryopreserved for later extraction or processed for DNA immediately, as outlined in the proposal. The serum DNA was isolated, purified, quantified, and aliquoted, as indicated in the tasks outlined for the proposal. These procedures were optimized to obtain maximum efficiency in isolating small amounts of DNA from the serum. Blood was processed to serum as indicated for Task 1. We have investigated various approaches in optimizing circulating DNA extraction from serum, and have an excellent procedure established. The focus has been to establish a good representative of the patients' circulating DNA in less than 200 ul serum. This task was critical, because the success of the downstream procedures will depend on whether sufficient amount of circulating DNA from only 200 ul of serum. In addition, we have been exploring the need to filter the serum for contaminating cells which may interfere in the assay. We have performed side-by-side studies of filtered and non-filtered serum from the same patients and normal donors. For methylation marker studies, filtration does not appear to be necessary. To study loss of heterozygosity of microsatellites, we use the CAE instrument and software for analysis. As indicated in Task 1 for the LOH studies, we established a panel of six microsatellite markers, D6S286, D8S262, D8S261, D9S171, D10S591, and D18S70, for assessment in patient samples. LOH of these markers were found to be most informative among others and are not found in normal tissue.

For Task 2, in developing and establishing serum methylated DNA markers, we have screened multiple tumor-related genes. Methylated and unmethylated DNA was assessed using specific markers by capillary array electrophoresis (CAE). In addition, we have developed realtime PCR analysis of methylated markers for validation of the results. This has been carried out with methylation-specific PCR primers labeled with specific TaqMan fluorescent dyes. In 85 evaluable patients, we have assessed approximately 25 AJCC Stages I, II, III patients, and 58 AJCC stage IV patients. Tumor-related genes were initially studied in cancer cell lines both for their DNA methylation status and then functionally verified via RT-PCR. Tumors were also studied to verify the clinical relevance of each gene screened. Of the genes we screened, RASSF1A, RARβ2, and GSTP-1 were established for assessment of serum samples. All three of these methylation markers are tumor-related genes and are known to be methylated in prostate tumor tissue and not in normal prostate tissue.

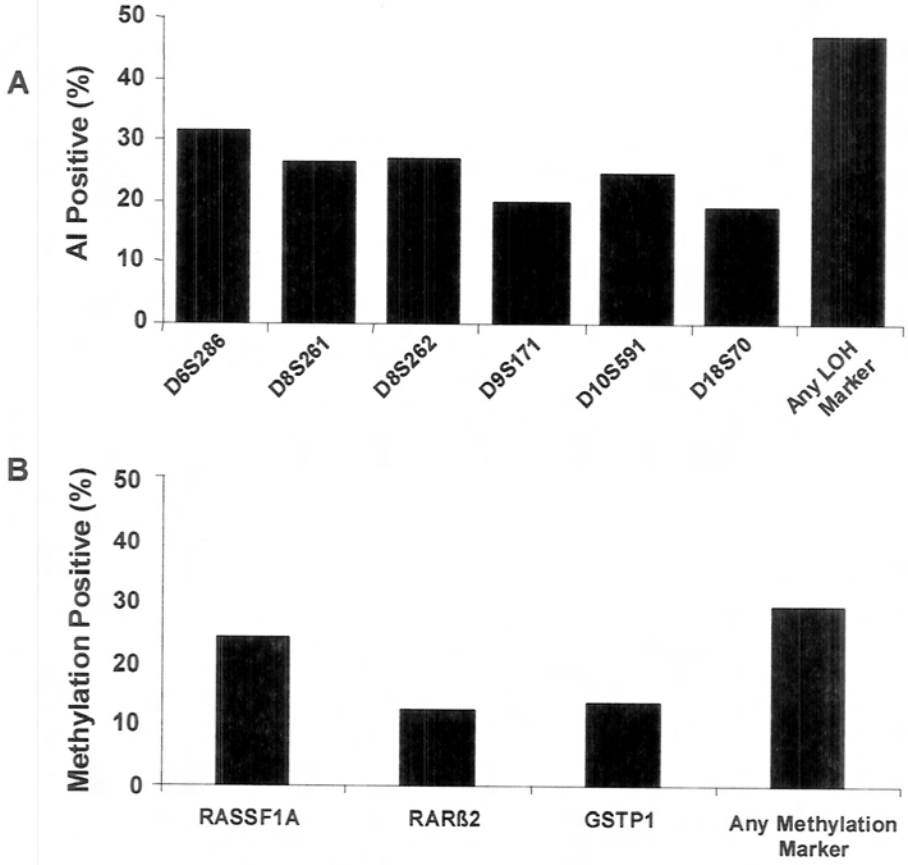
Of the patients accrued for serum samples, we were only able to obtain a small number of tumor tissues due to difficulties coordinating

among centers and hospitals and circumventing regulations and restrictions. Of the tumor samples we obtained, originally planned for Task 3, we performed micro-dissection using LCM and analyzed the tumors to establish the methylation and LOH marker panels.

Using the six LOH marker panel, AI in serum circulating tumor-related DNA were evaluated in the patient samples. The most common AI was detected with D6S286 microsatellite marker, which was detected in 14 of 45 (31%) informative patient samples. D8S262 was detected in 11 of 41 (27%), D8S261 was detected in 7 of 27 (26%), D10S591 was detected in 11 of 45 (24%), D9S171 was detected in 9 of 46 (20%), and D18S70 was detected in 11 of 58 (19%) (Figure 1A). The informative patient samples demonstrating AI for ≥1 marker was 47%, while healthy male donors (0%, n=40) did not have these specific LOH markers circulating in their sera.

The three marker gene methylation panel yielded 28% PCa detection rate in the 83 patient samples. RASSF1A was detected in 24%, RARB2 was detected in 12%, and GSTP1 was detected in 13% of patient samples (Figure 1B). Again, healthy male donors did not have these markers circulating in their sera as assessed by our assay.

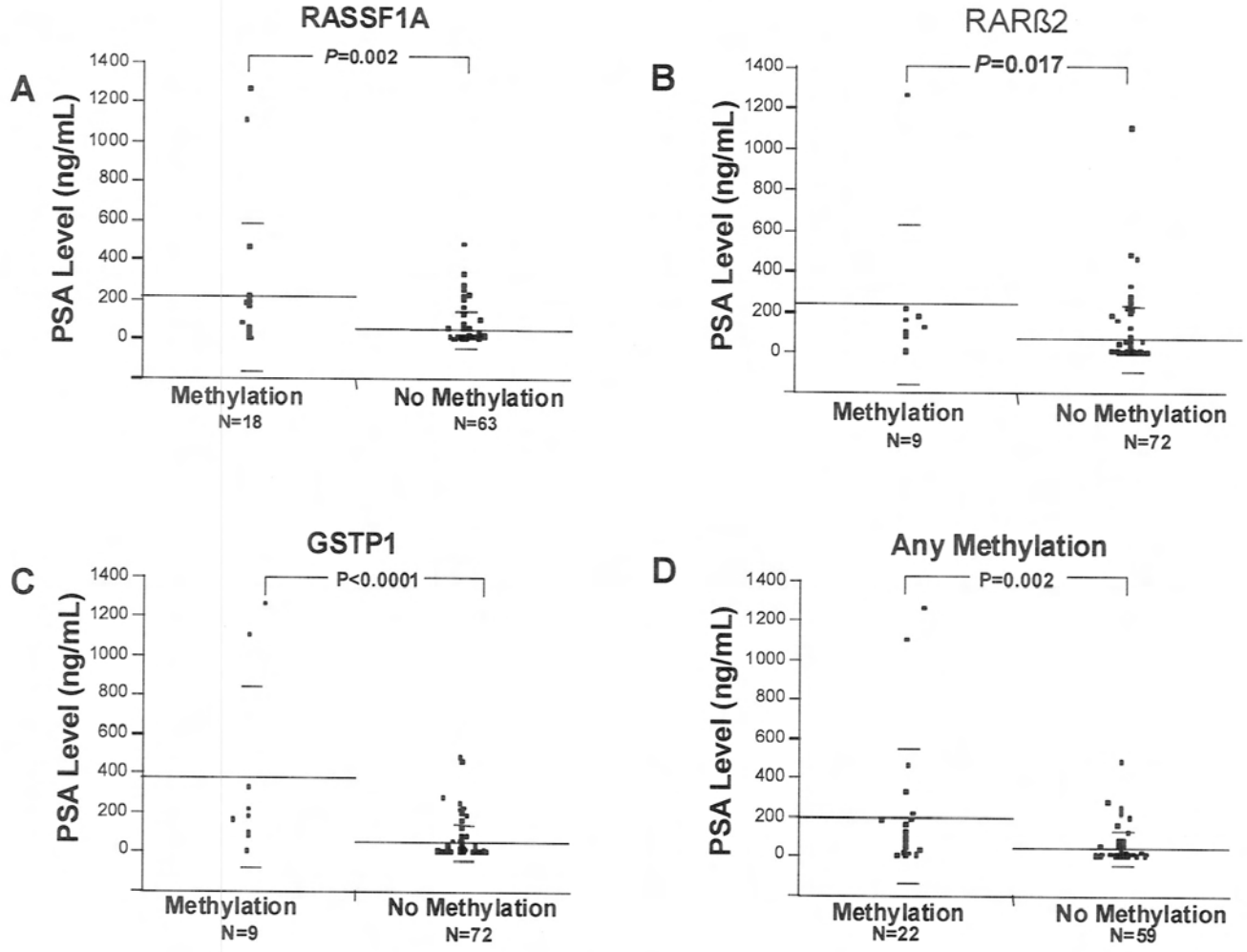
Figure 1



Circulating LOH and Methylated DNA in PCa patient serum were correlated to PSA levels. Serum PSA data was collected for 81 of the 83 patients accrued. No significant differences were seen in the serum PSA value between patients with AI of the microsatellites. The mean serum PSA level in patients with hypermethylated tumor-related genes was

significantly higher than in those not having hypermethylated genes (Figure 2). Similarly, patients with ≥1 hypermethylated tumor-related gene had serum PSA values significantly higher than patients without circulating hypermethylated DNA (P=0.002; Figure 2).

Figure 2



Primary PCa tumor Gleason score was also correlated with AI and methylation status. The Gleason score was available in 70 (84%) of 83 patients. There were no significant differences between the mean Gleason score in patients with AI of D9S171, D8S261, D6S286, D8S262, and D18S70 and that of heterozygous patients. The mean PCa Gleason score in patients with hypermethylated *RASSF1A*, *RARβ2*, and *GSTP1* was significantly higher (*P*=0.017, *P*=0.042, and *P*=0.016, respectively) than in patients without hypermethylated DNA. Patients with ≥1 hypermethylated circulating tumor-related gene had tumors with significantly higher Gleason scores than patients without hypermethylated DNA (*P*=0.019; **Table 1**).

Table 1. Serum Methylation and AI Biomarkers vs. Gleason Score

DNA Marker		GS	
		Mean (n)	*P* value
RASSF1A	Methylated	8.1 (17)	0.017
	Unmethylated	7.3 (53)	
RARβ2	Methylated	8.2 (9)	0.042
	Unmethylated	7.3 (61)	
GSTP1	Methylated	8.3 (9)	0.016
	Unmethylated	7.3 (61)	
Any Methylation	Methylated	8 (20)	0.019
	Unmethylated	7.2 (50)	
D6S286	AI	7.3 (12)	N. S.
	R	7.3 (26)	
D8S261	AI	8.2 (6)	N. S.
	R	7.5 (18)	
D8S262	AI	6.9 (9)	N. S.
	R	7.6 (23)	
D9S171	AI	7.8 (8)	N. S.
	R	7.6 (29)	
D10S591	AI	7.3 (9)	N. S.
	R	7.7 (28)	
D18S70	AI	7.2 (10)	N. S.
	R	7.6 (42)	
Any LOH	AI	7.5 (33)	N. S.
	R	7.4 (35)	

N.S. - Not significant

Detection of circulating methylated tumor-related DNA was also correlated with AJCC PCa stage. Circulating methylated DNA of *RARβ2* and *GSTP1* in serum was more common with increasing AJCC stage. Stage IV patients showed significantly more frequent hypermethylation of *RARβ2* and

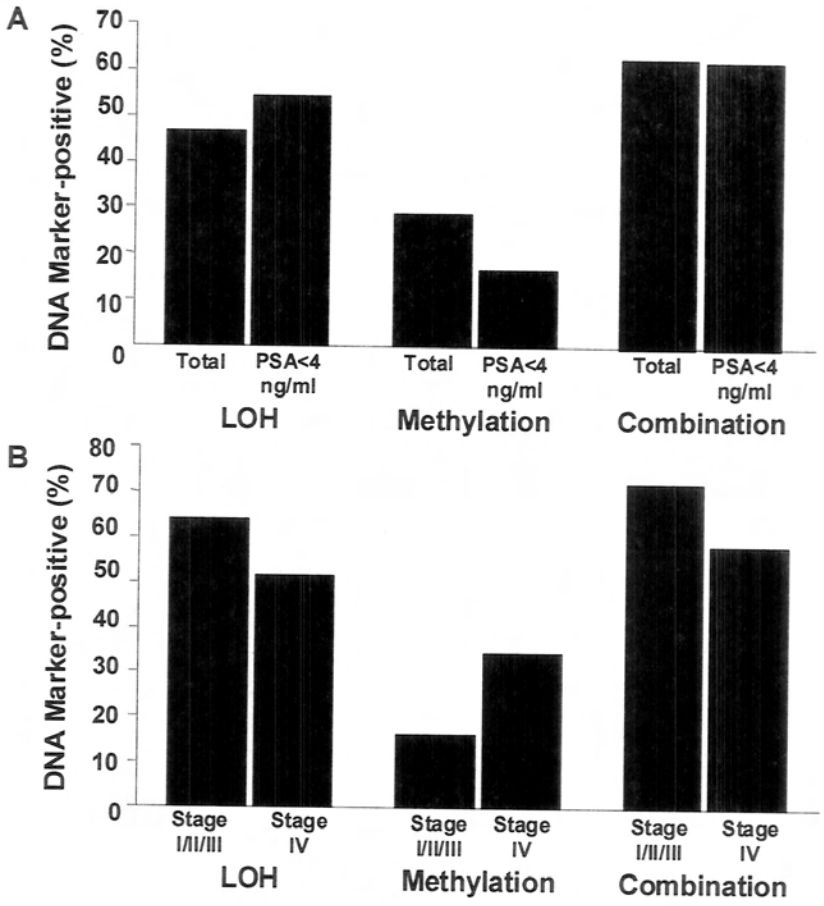
GSTP1 than stage I, II, and III patients (P=0.027 and P=0.019, respectively). Circulating methylated DNA of *RARβ2* and *GSTP1* was not seen in stage I, II, or III patients (**Table 2**).

Table 2. Methylation status and AJCC stage

Serum DNA Marker	Methylation Status	Stages I-III	Stage IV	*P* Value
		n=25	n=58	
RASSF1A	Methylated	4 (16%)	16 (28%)	N. S.
	Not methylated	21 (84%)	42 (72%)	
RAR β2	Methylated	0 (0%)	10 (17%)	0.027
	Not methylated	25 (100%)	48 (83%)	
GSTP1	Methylated	0 (0%)	11 (19%)	0.019
	Not methylated	25 (100%)	47 (8%)	
Any Methylation	Methylated	4 (16%)	20 (35%)	N. S.
	Not methylated	21 (84%)	38 (66%)	

Neither LOH nor hypermethylated DNA markers alone were able to identify all patients with PCa. By combining the two circulating DNA assays, the number of PCa patients positive for ≥1 methylation or LOH marker increased to 52 of 83 (63%). Of the 81 patients with available serum PSA (closest analysis to blood draw for DNA assays), 24 (30%) had values considered to be at low risk for a PCa diagnosis (<4 ng/ml). By combining the MSP and LOH assays, we identified 15 of 24 (63%) PCa cases not detected by PSA level alone (**Figure 3A**). The dual platform multimarker assays detected circulating tumor-related DNA with similar sensitivity, regardless of AJCC stage. Methylation or LOH of circulating tumor-related DNA was detected in 18 of 25 (72%) stage I/II/III and 34 of 58 (59%) stage IV PCa patients (**Figure 3B**).

Figure 3



The accrual of serial blood from patients receiving radiation therapy has been very limited due to the lengthy process of treatment and obtaining physicians' participation for the length of time. We were not able to collect any sets of serum with meaningful treatment regimen points. Some of the major issues were that patients would decide to switch treatment, become non-compliant to the regimens, or refuse to provide blood as requested. Therefore, we are unable to report any meaningful results on Task 4.d & e.

KEY RESEARCH ACCOMPLISHMENTS

1. PCA patients' accrual for blood.
2. Normal healthy male donors' sera accrual for blood.
3. Blood is processed for serum and cryopreserved.
4. Blood is processed for leukocytes as normal genomic controls.
5. Serum DNA is extracted, purified, and quantified.
6. DNA is isolated, quantitated and cryopreserved.
7. DNA is processed from leukocytes.
8. DNA LOH and methylation biomarkers developed and validated for sensitivity and specificity.
9. Tumor and cell line DNA screened for LOH and methylation biomarkers.
10. LOH marker and gene hypermethylation marker panels established.
11. Assessment assays optimized and standardized.
12. Serum DNA assessed using the LOH marker panel.
13. Serum DNA assessed for methylation marker panel.
14. Data from serum DNA markers correlated with patients' clinical status and prognostic factors.
15. Statistical analysis of marker correlation to clinical status and known prostate prognostic factors.
16. Identification of significant correlation of serum DNA markers with patients' Gleason Score and stage of disease.
17. Submitted findings for publication. Manuscript in revision (*Clinical Chemistry*)

REPORTABLE OUTCOMES

Reportable outcomes were limited during the funding period of this grant. Currently, a manuscript was submitted and is in the final review stage for publication. A second manuscript has also been prepared. A provisional patent was submitted for the use of LOH and methylation circulating DNA biomarkers in blood.

CONCLUSIONS

During the award years, we have been accruing patients to reach our objectives from three clinical sites. The studies progressed slowly forward in terms of patient and sample accrual. We were more successful in obtaining serum than tumor tissues. Serum collected from patients has been assessed on two different types of DNA biomarkers (LOH and methylation). The studies demonstrate that both types of biomarkers can be used to detect PCa in patient serum, and that these markers are not found in normal donor serum. The biostatistical analysis of the data generated indicates that there is a significant correlation of the biomarkers with disease status and prognostic factors. The serum assay produced through this award with both AI and methylation markers not only complement the current PSA serum assay but may surpass it by yielding additional information in regards to how to best manage the patient's disease.

REFERENCES

A Multimarker Circulating DNA Assay for Assessing Prostate Cancer Patients' Blood, Eiji Sunami, Masaru Shinozaki, Celestia S. Higano, Robert Wollman, Tanya B. Dorff, Steven J. Tucker, Steve R. Martinez, Frederick R. Singer, Dave S. B. Hoon, Clinical Chemistry, 2008.(Revision Submitted)

LIST OF PERSONNEL

Name	Institutional Affiliation(s)	Title(s)
Dave S.B. Hoon, PhD	John Wayne Cancer Institute	Director
Frederick R. Singer, MD FACS	John Wayne Cancer Institute	Director
Robert C. Wollman, MD	Saint John's Health Center	Medical Director
Celestia Higano, MD	Univ. of WA/Fred Hutchinson Cancer Research Center	Associate Professor
Sojun Hoshimoto, MD, PhD	John Wayne Cancer Institute	Postdoctoral Researcher
Norihiko Narita, MD, PhD	John Wayne Cancer Institute	Postdoctoral Researcher
Sharon Huang, PhD	John Wayne Cancer Institute	Research Associate
Stella Lam, BS	John Wayne Cancer Institute	Research Associate